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Definition of the Molecular Mechanisms Which Distinguish between Selective Estrogen Receptor Modulators (SERMs) and Full Antiestrogens

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Tamoxifen, a SERM (Selective Estrogen Receptor Modulator), is the most commonly used endocrine treatment for all stages of breast cancer. However, progression from tamoxifen sensitivity to tamoxifen resistance occurs in a substantial portion of the tumors. Full antiestrogens, such as ICI 182,780, are currently used as the second line therapy after failure of long-term tamoxifen therapy. To facilitate the design and characterization of more appropriate therapeutic agents for endocrine therapy of breast cancer, it is very important to understand the functional mechanisms that distinguish full antiestrogens from SERMs. It has been shown that estrogen receptor (ER) can recruit corepressors NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors) in the presence of tamoxifen, suggesting a possible role of N-CoR/SMRT in mediating the antagonist activity of tamoxifen. However, it is not clear if apo-ER or ICI 182,780-bound ER can recruit NCoR/SMRT or other corepressors. To investigate the possible involvement of different corepressors in the actions of different antiestrogens and unliganded ER, we have constructed a focused phage display library which contains the "CoRNR box" motif, a binding site important for N-CoR/SMRT to interact with the nuclear receptors. In this report, we have shown that screening of the CoRNR box library with ER treated with no hormone or different antiestrogens led to the isolation of peptides that differentially interact with apo-ER, tamoxifen-bound ER, or ICI 182,780-bound ER. These interactions observed *in vitro* have also been confirmed *in vivo* using a mammalian two-hybrid assay. Using a series of ER mutants, we were able to show that these CoRNR box-containing peptides have different binding characteristics from the peptides that contain the coactivator LXXLL motif. These peptides can be used to probe the conformational changes of ER induced by different antiestrogens and will be valuable for the design of screens for novel ER-antagonists.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Tamoxifen, ICI 182,780, estrogen receptor, corepressor, NCoR, SMRT, proteomics, phage display

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Introduction

The antiestrogen tamoxifen is the most commonly used endocrine treatment for all stages of breast cancer. Tamoxifen is a SERM (Selective Estrogen Receptor Modulator) which can act as an estrogen or an antiestrogen depending on the tissues in which it operates. Initially, tamoxifen may have profound effects on the survival and proliferation of breast cancer cells, but progressively, resistance emerges through mechanisms that are only partially understood. Some pure antiestrogens lack cross-resistance with tamoxifen, thus makes them good candidates as second-line therapy for patients who develop tamoxifen resistance and also suggests that the mechanisms underlying the actions of SERMs and pure antagonists are very different. This proposal investigates the possible involvement of different corepressor proteins in mediating the actions of different classes of antiestrogens. To probe the changes in the structure of the estrogen receptor (ER) upon binding of different antiestrogens, a focused phage displayed peptide library which expresses peptides containing a "CoRNR box" motif, important for corepressor to bind nuclear receptor, was made. Peptides that differentially interact with ER occupied by different antiestrogens were obtained. These peptides had provided a better understanding of actions of different antiestrogens and the assay which detects the peptide-ER interaction can be used as a sensitive and efficient system to screen for novel antiestrogens. During the course of the project in the last year, we have also used the technology we developed in studying estrogen receptor to examine the interaction of corepressor and retinoic acid receptor (RAR) and have brought forward more fundamental questions regarding nuclear receptor-corepressor interaction and its implication in breast cancer treatment.

Body

Screening the CoRNR box library with apo-ER or antiestrogen-bound ER has led to the isolation of pepitdes which interact with ER only in the absence of hormone or in the presence of antiestrogens. Importantly, none of these CoRNR peptides obtained interact with estrogen-bound ER, suggetsing that ER adopts very different conformations when treated with estrogen or antiestrogens. These peptides thus serve as a tool for understanding the molecular mechanisms of ER actions.

- **Enhanced transcriptional activity by a mutant ER which is unable to bind to the CoRNR box-containing peptides**

In our 2003 annual summary, we had reported the identification of peptides which interact differentially with apo-ER or ER treated with tamoxifen or ICI 182,780 by screening the "CoRNR box" phage displayed peptide library. Using a series of ERα mutants, we found that disruption of helices 3 and 5, which have been shown to be important for corepressor binding for PPAR, TR, and RXR (1, 2), had a marked effect on CoRNR box peptide binding. The results indicate that the CoRNR box peptides identified in this study can function as probes of antagonist-induced changes in ERα conformation. To determine whether the surfaces on the receptor with which these peptides interact could have a specific regulatory function, we compared the transcriptional activity of wild-type ERα (ER-WT) with the ER mutant in a reconstituted transcriptional system in transfected cells. One mutant, ER-L372R, which lost the ability to interact with CoRNR box-containing peptides without affecting its binding to LXXLL motif-containing peptides, exhibited enhanced transcriptional activity. As shown in Figure 1A, the constitutive transcriptional activity of ER-L372R was considerably lower than that displayed by the wild-type estrogen receptor. This effect is likely due to a general consequence of disturbances of the ER-LBD surface since other mutations in ERα helix 12 analyzed in the same manner also exhibit a lower level of constitutive activity. Tamoxifen manifests significant partial agonist activity in HepG2 cells expressing exogenous wild-type ERα, the magnitude of which is increased by a maximal 3-fold when assayed in cells expressing the L372R mutant (Figure 1C). In this cell system, the pure antiestrogen ICI 182,780 functions as an inverse agonist and in the absence of added agonist it caused a 90% repression of the basal transcriptional activity of the 3xERE-TATA-Luc promoter in cells expressing wild-type ERα. This repression activity is decreased by a maximal 4-fold when a similar assay is performed in cells expressing the ER-L372R mutant (Figure 1D). The most dramatic and unexpected result observed, however, was that estradiol-stimulated transcriptional activity of the ER-L372R mutant was greatly enhanced when compared to wild-type ER (Figure 1B). This observed increase in the efficacy of estradiol was most dramatic when 10 ng of the ER-L372R expression vector was used (11-fold more active than wild-type ER). Transfection of higher concentrations of ER-L372 led to a progressive decrease in estradiol-dependent transcriptional activity, possibly due to sequestration of limiting transcription factors by overexpression of this highly active mutant receptor. Similar results were observed using a C3-Luc reporter which contains a *bona fide* estrogen responsive human C3 complement promoter (data not shown). These results suggest that amino acid residue L372 is contained within a region which functions as a negative regulatory surface for ERα.

- **Receptor interacting domains of NCoR and SMRT interact with RAR or TR but not with ER**

In our 2003 annual summary, we have shown that the CoRNR box peptides identified in our screen interact with ERα or ERβ in the absence of hormone or in the presence of tamoxifen or ICI 182,780 (Figure 2A, 2B). Four CoRNR box-containing peptides derived from the receptor interacting domains (IDs) of NCoR/SMRT were also produced as Gal4-DBD-peptide fusions and tested in the same mammalian two-hybrid assay. Surprisingly, although these peptides derived from NCoR/SMRT IDs were capable of interacting with apo-TRβ (thyroid hormone receptor) or apo-RARα (retinoic acid receptor) and these interactions were disrupted upon addition of thyroid hormone (T3) or all-*trans*-retinoic acid (ATRA) (Figure 2C, 2D), we were unable to demonstrate an interaction of ERα or ERβ with any of these previously defined CoRNR box peptides found in NCoR/SMRT either in the absence of hormone or in the presence of antiestrogens (Figure 2A, 2B). It is possible that the ER-interacting domains within NCoR/SMRT may lie elsewhere. Alternatively, ER-binding corepressors may be different from the corepressors (NCoR/SMRT) bound to TR or RAR. One recent study has shown that a dominant-negative NCoR, which blocks corepressor activity by retaining receptor interaction domains while lacking repressor domains, can relieve transcriptional repression mediated by RAR but does not alter the ability of tamoxifen to inhibit ER-mediated transcription in MCF-7D breast cancer cells (3). This supports the hypothesis that corepresors other than NCoR/SMRT are involved in the tamoxifen-mediated antagonist activity.

- **Retinoic acid receptor and breast cancer**

Retinoids have been reported to inhibit the growth of breast cancer cell lines in culture and to reduce breast tumor growth in animal models (4, 5). The retinoid signals are mediated by the retinoic acid receptor (RAR) and retinoid X receptor (RXR). Both RAR and RXR are encoded by three distinct genes designated α, β, and γ. RAR and RXR are ligand activated transcription factors that regulate the transcription of target genes by binding to specific retinoic acid response elements (RAREs) located in the promoter region. In the absence of ligand, RAR/RXR are assoicated with a corepressor complex which include NCoR/SMRT. Upon ligand binding, NCoR/SMRT is displaced from RAR/RXR/RARE and coactivators are recruited to increase transcription. One of the target genes of RAR/RXR is RARβ. RARβ is highly expressed in normal mammary epithelial cells, but is down-regulated in breast cancer cells (6, 7). One possible mechanism for the reduced RARβ expression in breast cancer cells is the overexpression of corepressors NCoR and/or SMRT, which in turn repress basal transcription of RARβ gene. Indeed, a dominant-negative NCoR which contains only receptor interaction domains but not repressive domains can relieve transcriptional repression mediated by RAR on a RARE-Luciferase construct in MCF-7D breast cancer cells (3). This has prompted us to explore the possibility of using CoRNR box peptides to disrupt the corepressor-RAR interactions and thus increase the expression of RARβ in breast cancer cells. To screen for peptides which will bind apo-RAR, we have expressed and purified human RARα using the Bac-to-Back baculovirus expression system from Invitrogen Life Technologies. The CoRNR box library we had previously made were screened against the RARα using phage displayed technology. After three rounds of panning, peptides which interact with apo-RARα were obtained and the peptide-RARα interactions were confirmed using mammalian two-hybrid assay. As shown in Figure 3, most of the peptides identified from this screen (RC peptides) interact with RAR in the absence of hormone and these pepitde-RARα interactions were abolished in the presence of ATRA

treatment. These peptides exhibit the same characteristices as the those of the four pepitdes which correspond to the receptor interaction domains (IDs) of NCoR or SMRT. Furthermore, expression of these RAR-binding CoRNR box peptides into HepG2 cells have profound effect in increasing basal expression of a RARE-Luciferase construct as shown in Figure 4. These peptides thus hold promise for restoring the expression level of RARβ in breast cancer cells.

Key Research Accomplishments

- Used the CoRNR peptides identified in the phage displayed screening to dissect the mechanism of ER actions
- Discovered that ER-specific CoRNR peptides have different characteristics from those of the CoRNR peptides derived from corepressors NCoR and SMRT
- Generated contruct for expression of human RARα in insect cells
- Expressed and purified human RARα for phage displayed library screen
- Identified apo-RAR-specific peptides that exhibit same binding characteristics as those of the CoRNR box-containing peptides derived from corepressors NCoR and SMRT
- Showed that overexpression of the RAR-specific CoRNR peptides can increase the basal transcription rate of a RARE-TK-Luc construct.

Reportable outcomes

Manuscripts:

- Chang C.-Y., Norris J.D., Jansen M., Huang H.-J., McDonnell D.P. (2003) Application of random peptide phage display to the study of nuclear hormone receptors. *Methods in Enzymology* 364: 118-142.
- Huang H.-J., Norris J.D., McDonnell D.P. (2002) Identification of a negative regulatory surface within estrogen receptor alpha provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. *Molecular Endocrinology*, 16: 1778-1792.

Meeting Abstracts:

- Huang H.-J., McDonnell D.P. (2003) Development of peptide antagonists that target retinoic acid receptor alpha-corepressor interactions. Poster presentation at the 85th Annual Meeting of the Endocrine Society, Philadelphia, PA.
- Huang H.-J., Norris J.D., McDonnell D.P. (2002) Identification of a negative regulatory surface within estrogen receptor alpha provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. Poster presentation at Keystone Symposia on Nuclear Receptor Superfamily, Snowbird, UT.

Conclusion

During the past year we have used the CoRNR box peptides identified in our ER screen to further dissect ER actions. The loss of the ability of ER helix 3/5 mutants to interact with these CoRNR box peptides led us to examine the transcriptional activity of these mutants. The dramatically enhanced activity of ER-L372R help identify a negative regulatory surface within ER. When these peptides were examined for interacting with RAR and TR, we found that NCoR/SMRT may not be the corepressors mediating the antagonist activity of tamoxifen or ICI 182,780.

In our 2003 annual summary, we had reported that expression of these ER-specific CoRNR peptides were ineffective in decreasing the antagonist activity of tamoxifen or ICI 182,780 in cells. We thus extend our study to examine the role of RAR in breast cancer and its potential therapeutic application using the peptides that target the RAR-corepressor interactions. Using the same phage displayed technology we developed with ER, peptides which specifically bind apo-RARα were obtained. Interestingly, trasfection of these RAR-specific peptides can increase the basal transcription of a RARE-Luciferase construct in HepG2 cells. Future experiments will examine the effect of transfection of these peptides on endogenous RARβ expression in breast cancer cells.

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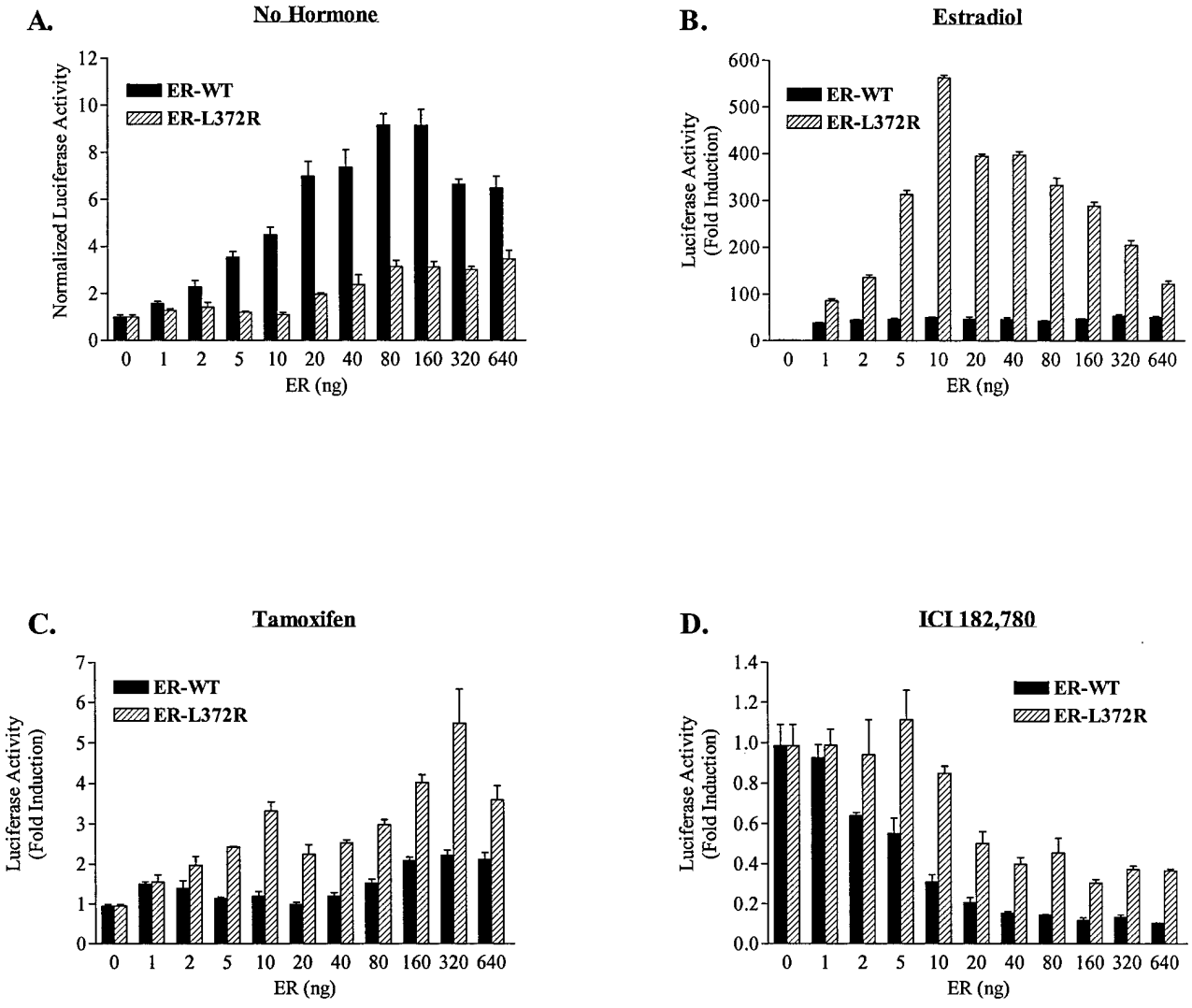

Figure 1. Analysis of the transcriptional activity of mutant ERα with amino acid substitution at Leucine 372. A-D, HepG2 cells were transfected with different amounts of wild-type or mutant ERα along with 3xERE-TATA-Luc reporter and the β-galactosidase control plasmid. Following transfection, cells were treated with different ligands (100 nM) for 24 h before harvested for luciferase and β-galactosidase activity. Data for panel A are presented as normalized luciferase activity, which was obtained by dividing the luciferase activity by β-galactosidase activity. Data for panels B, C, and D are presented as fold induction, which was obtained by dividing the normalized luciferase activity in the presence of ligand by that in the absence of ligand.

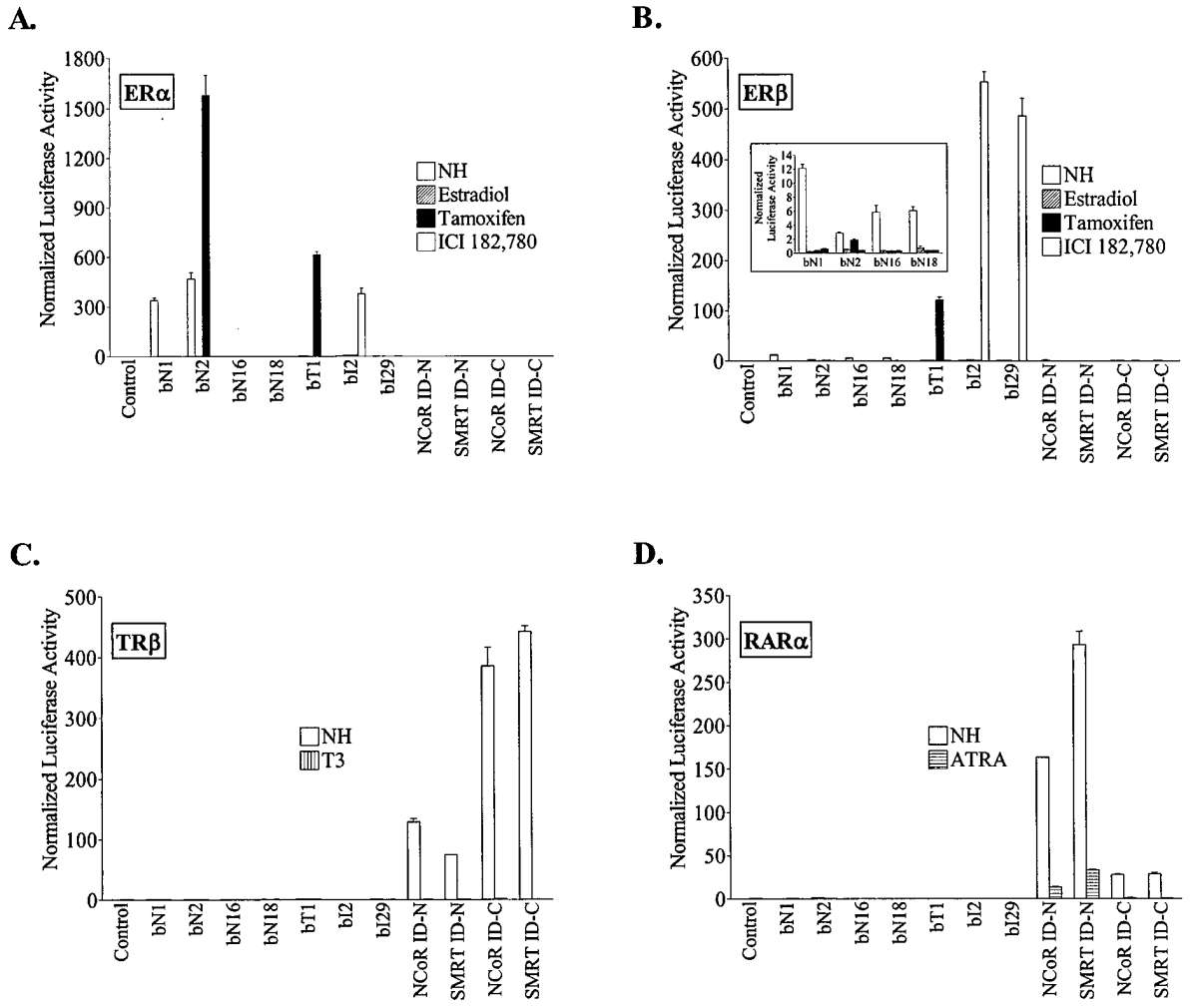

Figure 2. Evaluation of the interaction between receptor and CoRNR box-containing peptides in mammalian cells. HepG2 cells were transiently transfected with expression vectors for the Gal4-DBD-peptide fusion protein, a Gal4 responsive luciferase reporter construct (5xGal4-TATA-Luc), the β-galactosidase control plasmid, and VP16-ERα (A), VP16-ERβ (B), VP16-TRβ (C), or VP16-RARα (D). Transfection of the Gal4-DBD alone is included as control. Following transfections, cells were treated with 100 nM of estrogen or anti-estrogens (A and B), 100 nM of T3 (C), or 100 nM of all-*trans*-retinoic acid (D) for 24 h and assayed for luciferase and β-galactosidase activity. Data are presented as normalized luciferase activity, which was obtained by dividing the luciferase activity by β-galactosidase activity. The inset in panel B magnifies the lower part of the luciferase activity to emphasize the hormone-specific interaction between ERβ and CoRNR box-containing peptides.

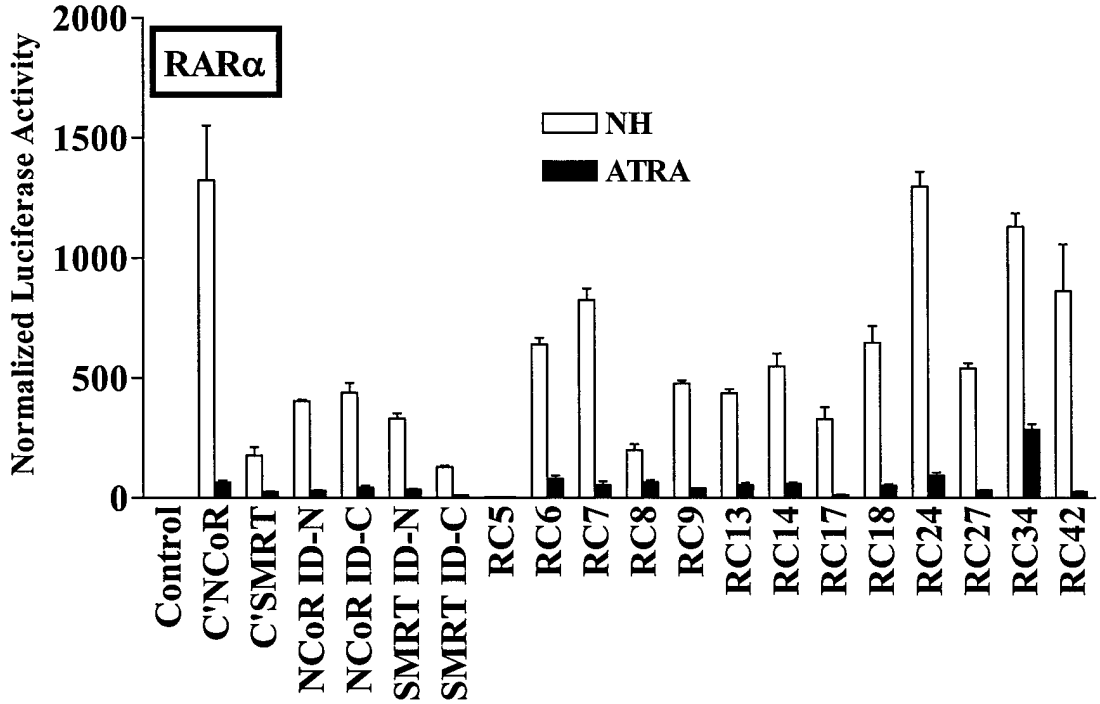

Figure 3. Evaluation of the interaction between RAR and CoRNR box-containing peptides in mammalian cells. HepG2 cells were transiently transfected with expression vectors for the Gal4-DBD-peptide fusion protein, a Gal4 responsive luciferase reporter construct (5xGal4-TATA-Luc), the β-galactosidase control plasmid, and VP16-RARα. Transfection of the Gal4-DBD alone is included as control. Following transfections, cells were treated with or without 1μM of all-*trans*-retinoic acid (ATRA) for 24 h and assayed for luciferase and β-galactosidase activity. Data are presented as normalized luciferase activity, which was obtained by dividing the luciferase activity by β-galactosidase activity.

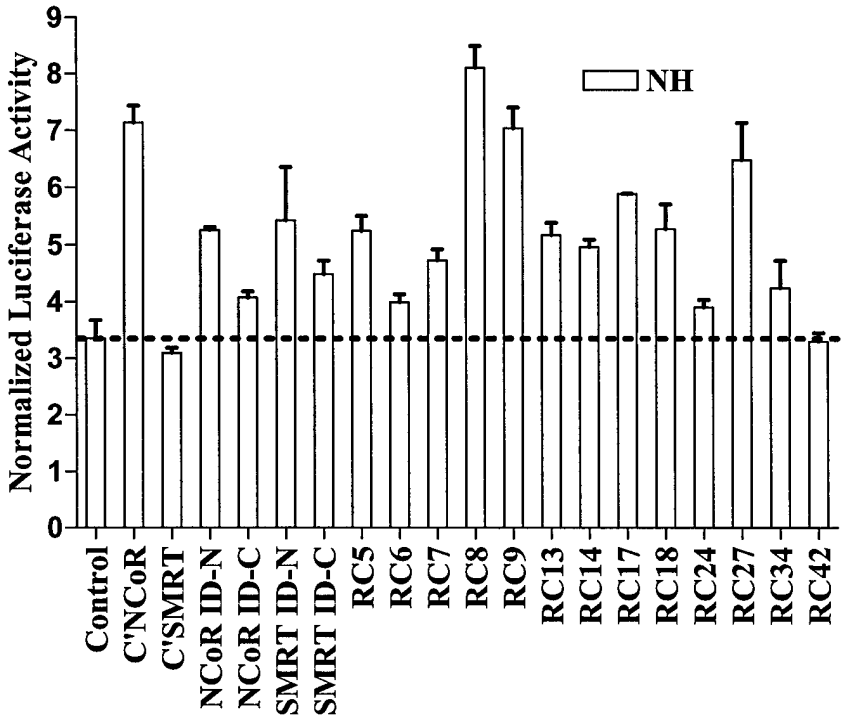

Figure 4. CoRNR box-containing peptides increase basal transcriptional activity of RARα when overexpressed in cells. HepG2 cells were transiently transfected with expression vectors for the Gal4-DBD-peptide fusion protein, RARα, a RARE-containing luciferase reporter construct (RARE-TK-Luc), and the β-galactosidase control plasmid. Transfection of the Gal4-DBD alone is included as control. 24 h after tansfections, cells were harvested and assayed for luciferase and β-galactosidase activity. Data are presented as normalized luciferase activity, which was obtained by dividing the luciferase activity by β-galactosidase activity.